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(54) Title: METHOD OF INDUCING APOPTOSIS IN A TARGET CELL

(57) Abstract

The present invention provides a method for inducing apoptosis in a target cell comprising introducing into a target cell an anti–sense RNA specific for an mRNA in the target cell. Vectors for delivering an anti–sense RNA into a target cell, and compositions comprising the vectors are also provided.

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METHOD OF INDUCING APOPTOSIS IN A TARGET CELL

SPECIFICATION

STATEMENT REGARDING FEDERALLY FUNDED RESEARCH

This invention was made with government support under CA 48654 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Many anti-cancer agents act by inducing apoptosis in cancer cells. This is particularly true of radiation therapy and treatment with DNA damaging agents. Induction of apoptosis by both radiation therapy and treatment with DNA damaging agents requires a functional p53 gene (Koshland (1993) Science 262:1953). One of the primary means of resistance to radiation and DNA damaging chemotherapeutic agents is through inactivation of p53. In addition, numerous DNA viruses encode inhibitors of p53-mediated apoptosis. In fact, the human papillomavirus (HPV) p53 inhibitors are thought to be involved in induction of cervical carcinoma by HPV (Kessis et al. (1993) Proc. Natl. Acad. Sci. USA 90:3988).

Recent studies have shown that many viruses are potent inducers of apoptosis, while other viruses encode potent inhibitors of apoptosis. Vaccinia virus (VV) encodes at least two inhibitors of apoptosis, the B13R gene and the E3L gene (Kettle et al. (1997) J. Gen. Virol. 78:677; Kibler et al. (1997) J. Virol. 71:1992; Lee et al. (1994) Virology 199:491). Deletion of either gene leads to induction of apoptosis in some cells. The B13R gene encodes a protease inhibitor that can block the cascade of caspases that are central to induction of apoptosis. The E3L gene encodes proteins that can bind to double-stranded (ds) RNA (Chang et al. (1992) Proc. Natl. Acad. Sci. 89:4825), which in the case of VV is a product of run-on transcription that occurs at late times post-infection. (Boone et al. (1979) J. Virol. 30:365). The

E3L gene products act as potent inhibitors of the interferon-inducible enzymes protein kinase PKR and 2',5' oligoadenylate synthetase (Beattie et al. (1995) J. Virol. 69:499). Both enzymes are induced by interferon in an inactive state and become activated by binding to dsRNA (Jacobs et al. (1996) Virology 219:339). When active, either enzyme can induce apoptosis in cells (Lee et al. (1994) Virology 199:491). Thus, either viral or synthetic dsRNA is a potent inducer of apoptosis in a wide variety of cells. Since interferon-treatment can induce synthesis of both PKR and 2',5' oligoadenylate synthetase, dsRNA induction of apoptosis can be significantly enhanced in many cases by treatment with interferon (Kibler, supra). dsRNA induction of apoptosis seems to be independent of p53, since dsRNA is a potent inducer of apoptosis in HeLa cells, which contain an HPV-encoded inhibitor of p53 (Kibler, supra). E3L appears to function upstream of B13R, since in some cells B13R can inhibit dsRNA-mediated apoptosis. dsRNA-induced apoptosis can also be inhibited by bcl-2, another well-characterized cellular inhibitor of apoptosis. Virus deleted for E3L is highly attenuated in human cells in culture, limiting the chances of spread of virus beyond the site of administration.

Run-on transcription at late times after VV-infection is the source of dsRNA in a VV-infected cell (Boone et al., supra). Transcription mediated by the virion DNA-dependent RNA polymerase terminates precisely at the ends of genes at early times post-infection. However, at late times post-infection, there is no precise termination of transcription. Instead, larger than gene-sized transcripts accumulate in VV-infected cells at late times post-infection. Since genes are encoded on both strands in VV and the genome is therefore transcribed in both directions, many of these long, late transcripts are complementary to one another, and form dsRNA at late times post-infection. It has been estimated that from 3-15% of the viral RNA transcribed at late times post-infection is capable of forming dsRNA (Boone et al., supra). The build-up of dsRNA at late times after infection with VV is effectively anti-sense RNA mediated formation of dsRNA in infected cells.

The accumulation of dsRNA during infection with VV can be controlled in a number of ways. Since late transcription requires viral DNA synthesis,

inhibitors of viral DNA synthesis, such as cytosine arabinoside (araC) prevent accumulation of viral dsRNA (Colby et al. (1971) J. Virol. 7:71). Temperature sensitive mutants in the subunits of the viral DNA polymerase are available, which are also altered at restrictive temperature in synthesis of viral DNA. The VV A18R gene inhibits processivity of the viral RNA polymerase (Simpson et al. (1994) J. Virol. 68:3642). Thus, mutations in A18R lead to longer than normal run-on transcripts and build-up of excess of dsRNA. The VV G2R gene increases processivity of the viral RNA polymerase (Condit et al. (1996) Virolosy 220:10). The drug IBT (DEFINE) has a similar effect. Mutation of G2R leads to smaller than normal transcripts and synthesis of limited amounts of dsRNA. Mutations in G2R can be complemented by treatment with IBT, and are thus IBT-dependent.

Because dsRNA is a potent inducer of apoptosis in a wide variety of cells, it provides a potential anti-cancer agent. Further, dsRNA induces suicide in a different manner than most other anti-cancer agents, and appears to induce suicide even in cancer cells that have become resistant to treatment with traditional anti-cancer agents. The obstacle to the use of dsRNA as an anti-cancer agent is that it causes both cancer cells and normal cells to commit suicide, leading to unacceptable side effects. The present invention overcomes this obstacle by providing a method of making dsRNA only in a target cell, thus allowing specific killing of target, e.g. cancer, cells while minimizing injury to normal cells.

SUMMARY OF THE INVENTION

The present invention provides a method for inducing apoptosis in a target cell. The method comprises introducing into a target cell an anti-sense RNA specific for an mRNA in the target cell under conditions whereby the anti-sense RNA binds to the mRNA to form dsRNA, resulting in apoptosis. In a preferred embodiment the target cell is a cancer cell.

In another embodiment, the present invention provides vectors for delivering an anti-sense RNA to a target cell. In a preferred embodiment the vector is a vaccinia virus (VV) vector in which the E3L and B13R genes have been deleted or

otherwise inactivated, and which comprises a nucleic acid encoding the anti-sense RNA operably linked to a promoter, preferably a VV early promoter. Compositions comprising the vector and a carrier are also provided.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for inducing apoptosis in a target cell. The present method comprises introducing into a target cell an anti-sense RNA that is specific for an mRNA in the target cell, under conditions whereby the anti-sense RNA binds to the mRNA to form dsRNA in the target cell, which results in apoptosis.

In a preferred embodiment, anti-sense RNA is introduced into a target cell by means of a vector containing a nucleic acid encoding the anti-sense RNA. Anti-sense RNA is defined herein as an RNA molecule that is sufficiently complementary to a selected mRNA in a target cell such that it binds to the mRNA to provide a dsRNA molecule. In a preferred embodiment, the anti-sense RNA comprises at least 50 base pairs of exact complementarity to the selected mRNA.

The present invention further provides vectors for delivering an anti-sense RNA to a target cell. In a preferred embodiment, the vector is a viral vector. Viral vectors that are not harmful to selected host cells or that have been rendered non-virulent and that can be manipulated to contain heterologous nucleic acids are well-known in the art and include, for example, poxvirus, adenovirus, papilloma virus, parvovirus, and vaccinia virus vectors. In a preferred embodiment, the viral vector is a vaccinia virus (VV) vector. In another preferred embodiment, the VV vector has been manipulated to inactivate VV genes that inhibit dsRNA-mediated apoptosis, namely the E3L and B13R genes.

The VV genes E3L and B13R are known to inhibit dsRNA-mediated apoptosis. Kibler et al. (1997) J. Virol. 71:1992, the disclosure of which is incorporated herein by reference, disclose that the E3L gene inhibits the dsRNA mediated induction of apoptosis. Deletion of the gene leads to potent induction of apoptosis by dsRNA synthesized at late times after infection with VV. Induction of

apoptosis by VV deleted of E3L can be augmented by pretreatment of cells with interferon, which induces synthesis of the enzymes PKR and 2', 5' oligoadenylate synthetase. Inactivation of the VV E3L gene can be achieved by standard manipulations known to those of ordinary skill in the art, for example by deletion of the E3L gene. Kibler et al., ibid., and Beattie et al. (1995) J. Virol. 69:499 disclose E3L-deleted VV. VV in which the E3L gene has been inactivated or deleted is known as VV Δ E3L. A Copenhagen strain VV in which the lacZ gene replaces E3L is known as cp VVΔE3L.

The VV B13R gene codes for a potent inhibitor (SPI-2/crmA) of the apoptosis-associated proteases, the caspases (Kettle et al., (1997) J. Gen. Virol. 78:677). Under some conditions, the endogenous B13R gene product can inhibit dsRNA mediated apoptosis. Accordingly, in the VV vectors of the present invention, the B13R gene is preferably inactivated. Inactivation of the VV B13R gene can be achieved by standard manipulations known to those of ordinary skill in the art, for example by deletion of the B13R gene. Also, some naturally occurring strains of VV, for example the Copenhagen strain (cpVV), contain natural deletions in B13R that inactivate the gene.

In a preferred embodiment of the present invention, the VV vector is a cpVV in which the E3L gene has been inactivated.

The VV vectors useful in the method of the present invention may also contain a deletion or other inactivation of the K3L gene. Kibler et al. (1997) J. Virol. 71:1992 disclose a K3L-deleted VV. The K3L gene can function as an inhibitor of the cellular enzyme PKR, which has been shown to be a mediator of dsRNA-induced apoptosis by Davies et al. (1993) J. Virol. 67:1688. For example, a cpVV variant in which the K3L gene has been replaced by an ecogpt selectable marker (encoding resistance to mycophenolic acid) is useful in the method of the present invention and has been designated cpVVΔK3L.

Recombinant virus in which genes such as E3L, B13R and K3L are inactivated are known in the art or may be prepared, for example, by co-infecting VV

permissive cells with cpVV ΔE3L and cpVV ΔK3L. Recombinant virus is identified as mycophenolic acid-resistant virus yielding blue plaques in the presence of X-gal (5-bromo-4-chloro-3-indolyl-β-galactosidase). Rabbit kidney RK-13 cells are permissive for VV that has been altered in its dsRNA, as disclosed by Beattie et al. (1996) Virus Genes 12:89, incorporated herein by reference.

In the above-described VV vectors, the E3L and B13R genes, and optionally the K3L gene, have been inactivated, thus deleting the VV inhibitors of dsRNA-mediated apoptosis. These deletions leave host cells susceptible to induction of apoptosis by viral dsRNA that is synthesized at late times after VV infection. The dsRNA accumulates in VV infected cells due to accumulation of longer than gene length, run-on transcripts at late times post-infection. Since genes in VV are encoded on both strands of the viral DNA, the long transcripts are often complementary.

In accordance with the present invention, apoptosis induced by viral dsRNA is prevented by treating target cells with cytosine arabinoside (araC), or by eliminating the VV G2R gene from the VV vector. Accordingly, apoptosis induced by the present vectors results only from dsRNA formed by antisense RNA provided by the vector, and mRNA contained in, and specific to, the target cell. By preventing induction of viral dsRNA, apoptosis of non-target cells is prevented.

AraC is a potent inhibitor of VV DNA synthesis and thus also of late gene expression. In addition, araC treatment allows continued synthesis and accumulation of early gene products, such as the anti-sense RNA, that are under the control of an early VV promoter. Since dsRNA in a VV infection is a product of late transcription, accumulation of undesired VV dsRNA is potently inhibited by treatment of target cells with araC.

Accumulation of long, run-on transcripts at late times post infection requires a functional viral G2R gene, as disclosed by Condit et al. (1996) Virology 220:10, incorporated herein by reference. Inactivation of the G2R gene results in a deficiency of late accumulation of viral dsRNA. Both deletion mutants and temperature sensitive mutants in G2R are known in the art. G2R mutants are dependent upon the drug isatin β-thiosemicarbazone (IBT) for growth. Thus mutation

in G2R can be recombined into cpVVΔE3L/ΔK3L by co-infection and selection for IBT-dependent, mycophenolic acid-resistant virus that yields blue plaques in the presence of X-gal.

In a preferred embodiment of the present invention, the VV vector contains inactivations or deletions of the E3L, B13R and G2R genes.

Certain cellular genes or systems are considered to be anti-apoptotic in that they inhibit dsRNA-induced apoptosis. These include bc1-2, NF-kB, P58 and dsRNA adenosine deaminase (ADAR). Various genes that inhibit the function of these anti-apoptotic genes or systems may be included in the VV vectors of the present invention.

The vectors of the present invention further contain a nucleic acid that encodes an anti-sense RNA. The anti-sense RNA is selected to be complementary to a mRNA that is specific for a target cell of interest. Accordingly, dsRNA is made, and apoptosis is induced, only in target cells that contain the specific mRNA. Selectivity of induction of apoptosis is thus determined by the identification of a species of mRNA that is specific to a particular target cell. Certain target cell specific mRNAs, for example tumor specific mRNAs, are known in the art, including for example the cervical carcinoma specific human papilloma virus E6 or E7 mRNAs (Fujii et al. (1995) Gynecol. Oncol. 58:210, incorporated herein by reference), the multidrug resistance (MDR) RNA (Lowenberg et al. (1998) Curr. Opin. Oncol. 10:31, incorporated herein by reference), and the thromboxane synthase mRNA in rapidly migrating glioma cells. Further, target cell specific mRNAs can be identified by methods known in the art, for example by subtractive hybridization, electronic subtraction, and differential display (DD). See, e.g., Poirier et al. (1997) Nucleic Acids Res. 25:913. DD reverse transcriptase polymerase chain reaction (RT-PCR) is preferred and described by Liang et al. (1982) Science 257:967. By DD RT-PCR, RNA is isolated from target cells, e.g. tumor cells, and non-target cells, e.g. normal cells. Complementary DNA (cDNA) is generated from the RNA and displayed, for example on polyacrylamide gets. The cDNAs that correspond to mRNA expressed

uniquely in target cells are isolated and characterized, for example by sequence analysis, to identify mRNA that is specific to the target cells.

An anti-sense RNA that is complementary to the target cell - specific mRNA is then determined. The anti-sense RNA must be sufficiently complementary to the selected target cell-specific mRNA such that it binds to the mRNA in the target cell to provide a dsRNA. In a preferred embodiment, the anti-sense RNA comprises at least 50 base pairs of exact complementarity to the selected mRNA.

DNA encoding RNA that is antisense (complementary) to the target cell-specific mRNA can be determined by the ordinarily skilled artisan and introduced into the vector of the invention by methods known in the art. For example, heterologous DNA can be inserted into VV at multiple possible incorporation sites. A plasmid containing the heterologous DNA flanked by DNA from a nonessential region of the VV genome may be used for recombining the heterologous DNA into the VV vector. Cultured cells are infected with the VV vector and transfected with the plasmid to incorporate the heterologous DNA into the VV vector by homologous recombination. For example, a useful plasmid is pMPE3LΔgpt described by Beattie et al. (1995) J. Virol. 69:499, the disclosure of which is incorporated herein by reference. This plasmid contains the E3L flanking arms for homologous recombination; the E3L right flanking arm contains the E3L promoter. In a preferred embodiment the VV vector is the modified VV vector described hereinabove in which the E3L gene has been deleted. Preferred host cells for homologous recombination include rabbit kidney RK-13 cells.

Recombinant viral vectors can be extracted from the host cells by standard methods, for example by rounds of freezing and thawing as described for example by Kibler et al., supra. In the resulting recombinant viral vector, the heterologous DNA encoding the antisense RNA is preferably under the control of the E3L promoter.

In accordance with the present method, the viral vectors are introduced into target cells under conditions whereby the heterologous DNA is then transcribed to provide an antisense RNA molecule. The antisense RNA molecule binds to a specific

mRNA in the target cell to provide dsRNA, the production of which results in apoptosis.

In a preferred embodiment, the vector is a VV vector, and the VV vector is introduced into the target cell by infection. Suitable target cells include cancer cells, virus-infected cells, cells infected with other agents, and uninfected cells. Preferred target cells include lung carcinoma cells, cervical carcinoma cells, and rapidly migrating brain cancer cells such as gliomas.

Introduction of the vector of the present invention into a target cell results in production of dsRNA and apoptosis of the target cell. Apoptosis of target cells can be assessed by methods known in the art, including for example DNA laddering, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assays, and DNA dye binding assays. In DNA laddering, characteristic nucleosome-sized cytoplasmic DNA indicates the presence of fragmented cytoplasmic DNA in the cytoplasm, and is used as a marker for apoptosis. In TUNEL staining of cell monolayers, fluorescein-linked digoxigenin antibodies are used to detect the location of deoxyuridine residues that have been attached by terminal transferase to free 3' OH groups of DNA. Brightly fluorescing cells indicate the presence of fragmented DNA. In DNA dye binding assays, extranuclear staining with a DNA-intercalating dye such as Hoechst 33258 is a marker for apoptosis. The foregoing methods are known in the art and disclosed, for example by Kibler et al., supra.

The present invention further provides a composition comprising a vector containing a nucleic acid encoding an anti-sense RNA specific for an mRNA in a target cell, and a carrier. The vector is preferably a VV vector. The term carrier as used herein includes any and all solvents, diluents, dispersion media, antibacterial and antifungal agents, microcapsules, liposomes, cationic lipid carriers, isotonic and absorption delaying agents, and the like.

The methods, vectors, and compositions of the present invention are useful for inducing apoptosis in a target cell. For example, the present invention can be used to cause apoptosis and therefore death of specific unwanted cells in vitro, for example in cell culture systems, and in vivo, for example in tumors. The vectors of

the present invention can be delivered to target cells in vivo by methods known in the art, including for example by intravenous, subcutaneous, ultramuscular, intratumor, intraperitoneal, and intraepidural injection, by topical administration, by inhalation, by depot injections or erodible implants, and by direct application to tissue surfaces, for example during surgery.

All references cited herein are incorporated by reference in their entirety.

The following examples further illustrate the present invention.

Example 1.

Materials and Methods

All engineered viruses are analyzed for the appropriate phenotypic characteristics (i.e., mycophenolic acid-resistance, X-gal staining, X-glc staining, IBT-dependence, etc.), using standard laboratory procedures. Correct insertion of genes is confirmed by PCR, by sequencing and by Western blot analysis. Virus stocks are grown in RK-13 cells, which have proven permissive for VV strains altered in their dsRNA modulatory genes. BHK and CEF cells have also proven permissive for VV strains altered in their dsRNA modulatory genes.

Cells are seeded in 35 mm or 60 mm dishes and infected at an MOI of 5 pfu/cell with the appropriate virus. Cells are treated with 10μg/ml araC at the time of infection and harvested for analysis at 12, 24 and 36 hours post-infection.

Three techniques are used to analyze apoptosis: 1) DNA laddering, 2) TUNEL, and 3) DNA dye binding assays.

DNA laddering utilizes accumulation of nucleosome sized cytoplasmic DNA as a marker for apoptosis. Briefly, cells are scraped into their medium, pelleted and washed in cold phosphate buffered saline (PBS). Cells are lysed in a buffer containing a mild detergent that does not disrupt nuclei. Nuclei are removed by centrifugation at 10,000 x g. The supernatant solution is extracted with phenol to remove proteins, and nucleic acids are concentrated by precipitation with ethanol. Precipitated nucleic acids are resuspended in Tris/EDTA (TE) and treated with

ribonuclease A. The sample is then separated by agarose gel electrophoresis and the nucleic acids are visualized with UV light after staining with ethidium bromide.

TUNEL staining of cell monolayers, which identifies degraded DNA, is also used as a marker for apoptosis. Briefly, cells monolayers are rinsed with PBS and fixed in place with a mixture of formaldehyde and glutaraldehyde. Fixed cells are permeabilized with detergent. The permeabilized cells are treated with terminal deoxynucleotide transferase and digoxigenin-dATP. Digoxigenin-dATP that has been added to the ends of fragmented DNA is visualized after incubation with fluorescently labeled anti-digoxigenin. Cells are visualized on an inverted fluorescence microscope.

Staining of cellular DNA with Hoechst 3328 effectively detects evidence of apoptosis. This procedure gives information as to the morphology of cells, including visualization of cytoplasmic apoptotic bodies and chromatin condensation.

Staining with acridine orange/ethidium bromide is also used to detect apoptotic cells.

Apoptosis can also be assayed by fluorescence activated cell sorting (FACS) analysis of propidium iodide stained cells. FACS analysis has the advantage of allowing quantitative determination of the percentage of cells undergoing apoptosis.

Example 2.

Inactivation of Cellular Inhibitors of dsRNA Induced Apoptosis

A number of cellular systems have been identified that can actually or potentially inhibit dsRNA-induced apoptosis. These include bcl-2, NF-kB, P58, and dsRNA adenosine deaminase (ADAR). On the other hand, induction of apoptosis by dsRNA appears to be p53 independent, since dsRNA can potently induce apoptosis in HeLa cells, which contain a papilloma virus inhibitor of p53. Various genes are inserted VV to inhibit function of these anti-apoptotic genes.

B cells engineered to express high levels of bcl-2 are resistant to induction of apoptosis by dsRNA. The genes for at least one known inhibitor of bcl-2 are inserted into VV, to counteract the potential anti-apoptotic effects of bcl-2. Bax, bcl-xs or bag expression from VV are tested to determine whether the bcl-2 inhibition of apoptosis by VV deleted for E3L can be counteracted. These genes are inserted into the E3L locus, under control of the highly efficient early E3L promoter. Briefly, the gene for one of the bcl-2 inhibitors described above is cloned into an E3L insertion vector. Cells are transfected with the vector DNA and infected with cpVV ΔE3L. Recombinant virus that has taken up the bcl-2 inhibitor gene by double homologous recombination is identified by loss of blue staining with X-gal. Correct insertion is confirmed by PCR, DNA sequence analysis and Western blot analysis. To determine if the inserted gene can inhibit bcl-2 function, induction of apoptosis by VV deleted for E3L either lacking or containing a bcl-2 inhibitory gene in B cells either expressing or lacking bcl-2 is compared.

Activated NF-kB has been shown to induce expression of survival genes that can block induction of apoptosis (Beg et al. (1996) Science 274:782). Since activated PKR can phosphorylate I-kB, and lead to activation of NF-kB, a gene coding for a non-phosphorylatable mutant of I-kB is inserted into VV. Non-phosphorylatable mutants of I-kB have been shown to block activation of NF-kB in a dominant manner. The gene for a non-phosphorylatable mutant of I-kB is inserted into the K3L locus of VV by double homologous recombination, and selection for resistance to 6-thioguanine, which is toxic. Incorporation of the gene for I-kB leads to deletion of *ecogpt* and resistance to 6-thioguanine.

To test function of the non-phosphorylatable mutant of I-kB, the ability of virus infection to block TNF-α induced apoptosis in the absence of cycloheximide is determined. Normally cycloheximide must be included in treatment of HeLa cells with TNF-α to prevent accumulation of survival gene products which are induced by TNF-α activation of NF-kB. TNF-α should be able to induce apoptosis in cells infected with VV engineered to express a non-phosphorylatable mutant of I-kB even in the absence of cycloheximide.

P58 is an endogenous cellular inhibitor of PKR that is expressed in many cells. P58 can bind to PKR and block its interaction with substrates. It is unclear if P58 can inhibit the dsRNA-mediated induction of apoptosis, but P58 expression can lead to transformation of NIH-3T3 cells. An endogenous protein inhibitor of P58 has recently been isolated and characterized. The corresponding gene has been cloned. This gene is expressed from the B13R locus under control of an E3L promoter. A *gus* gene is inserted into the B13R locus of cpVV to allow for screening of recombinants. The gene for P58 inhibitor is inserted by double homologous recombination, using loss of blue staining in the presence of X-glc to identify recombinants.

dsRNA adenosine deaminase (ADAR) is a developmentally regulated, cell cycle regulated, interferon inducible enzyme that can inactivate dsRNA in cells. ADAR can deaminate adenosine in dsRNA to inosine, with the effect of denaturing the strands of dsRNA. At least some of the failures of anti-sense technology have been attributed to expression of ADAR in cells. However, the effects of ADAR can be overcome in cells, since dsRNA is a potent inducer of apoptosis. The majority of ADAR activity is present in the nucleus of cells, and thus should not interfere with dsRNA induced in cells by VV, which replicates solely in the cytoplasm. ADAR effects on anti-sense induction of dsRNA are assayed using an assay for adenosine deamination disclosed by Norse et al. (1997) Biochemistry 36:8429, incorporated herein by reference. A region of the E3L gene that has homology to ADAR is assessed to determine if it can act as a dominant negative effector of ADAR. This region is inserted into cpVVΔE3L. This region of E3L does not inhibit the dsRNA-induction of apoptosis.

Example 3.

Use of Anti-Sense RNA to a Specific mRNA to Induce Apoptosis in a Target Cell

Kibler et al. (1997) J. Virol 71:1992 have shown that dsRNA is a potent inducer of apoptosis in HeLa cells, despite their loss of functional p53 activity. HeLa cells are used to demonstrate that anti-sense to a specific RNA can be used to specifically kill cancer cells.

Apoptosis is induced in cells that have been transfected with either a neomycin or a zeocin resistance marker and a color detection system. These cells are prepared by transfection of HeLa cells with either pcDNA3.1/Neo/GFP (green fluorescent protein) or pcDNA3.1/Zeo/BFP (blue fluorescent protein), and selection with the appropriate antibiotic. Genes encoding anti-sense and sense *neo* are then inserted into pMPΔE3L. This plasmid allows replacement of the *lacZ* gene in the E3L locus of cpVVΔE3L/ΔK3L with sense or anti-sense *neo*. Viruses that have had *lacZ* replaced by neo are identified by loss of blue staining with X-gal. Proper virus construction is confirmed by PCR and nucleotide sequence analysis. These viruses are then used to infect araC-treated HeLa pcDNA3.1/Neo/GFP and HeLa pcDNA3.1/Zeo/BFP cells, which are analyzed for induction of apoptosis. If anti-sense RNA induces dsRNA-mediated apoptosis in HeLa cells, apoptosis occurs in HeLa pcDNA3.1/Neo/GFP cells infected with VV anti-sense *neo*, but not in cells infected with VV sense *neo*. Induction of apoptosis in HeLa pcDNA3.1/Zeo/BFP cells infected with either virus is not expected. The effect of pre-treatment with interferon-αA/D (kindly provided by Hoffmann-LaRoche) on induction of apoptosis is investigated.

The maximum amount of dsRNA needed to induce apoptosis in cells has been estimated. It is estimated that HeLa cells infected with VVΔE3L that are undergoing apoptosis contain approximately 10^{-16} g dsRNA/cell. This amount of dsRNA is enough to lead to complete activation of PKR in infected cells. Since the average size of a molecule of dsRNA in a VV infected cell is 1,000 bp, 10^{-16} g dsRNA/cell corresponds to approximately 100 molecules of dsRNA/cell. Thus, this is likely the upper limit of the amount of dsRNA that would need to be formed in cells expressing an anti-sense construct. Since an average cell contains about 10^{-13} g of mRNA, then if a mRNA is present at about 0.1% abundance, enough dsRNA should be formed with an

anti-sense construct to obtain 10^{-16} g of dsRNA/cell. Thus only a moderately abundant tumor specific mRNA is necessary for the present method.

The minimum amount of dsRNA that is actually necessary to induce apoptosis in cells is determined in one of two ways. 1) VSV DI-011 is a defective interfering particle of vesicular stomatitis virus that contains a pure dsRNA genome (Marcus et al. (1977) Nature 266:815). Since VSV DI-011 cannot replicate by itself in cells, when a cell is infected with a single particle of VSV DI-011, a single molecule of dsRNA is deposited into the cytoplasm of the cell. One can simply ask how many particles of VSV DI-011 must infect a cell to induce apoptosis in that cell. Alternatively, 2) anti-sense to *neo* is expressed under a controllable VV promotor as described by Ward et al. (1995) Proc. Nat'l. Acad. Sci. USA 92:6773. Synthesis of varying amounts of anti-sense *neo* is induced, and the minimal amount that is necessary to induce apoptosis is determined. The amount of dsRNA present in the cells is measured by isolating RNA, treating with a single-stranded RNA specific RNase, and then determining the amount of anti-sense and sense *neo* that is RNase resistant by quantitative RT-PCR, using known amounts of *in vitro* transcripts as standards.

Apoptosis is induced with anti-sense to a potentially cancer specific mRNA. MDR is often over-expressed in chemotherapy resistant cancers (Cole et al. (1994) Cancer Res. 54:5902). Anti-sense to MDR is used to induce apoptosis in HeLa/MDR cells. The ability to induce apoptosis with endogenous cervical carcinoma specific mRNAs, the human papilloma virus E6 and E7 mRNAs, is also determined.

Thromboxane synthase mRNA is specific for rapidly migrating but not slowly migrating glioma cells. This mRNA was isolated by selecting for rapidly migrating and slowly migrating glioma cells and then screening for differently expressed genes by DD RT-PCR. Basically, RNA from both cells was amplified by RT-PCR using four different anchored oligo-dT primers and five different pools of upstream primers. RT-PCR products were separated by gel electorphoresis and bands over-represented in the sample from one cell type were cloned and sequenced. Differential expression was confirmed by Northern blot analysis and quantitative RT-PCR.

To determine whether dsRNA can induce apoptosis in glioma cells, cells are infected with cpVVΔE3L or wtVV and differences in induction of apoptosis are assessed, or dsRNA is transfected directly into these cells. The ability of interferon-treatment to maximize dsRNA-induction of apoptosis in these cells is also determined. Anti-sense RNA to thromboxane synthase mRNA is assessed for its ability to specifically induce apoptosis in rapidly migrating glioma cells. It is known that dsRNA can induce apoptosis in several different cell lines, including HeLa cells, murine B cells, L cells, RK-13 cells, vero cells, and rat-1 cells. Virus modified to include inhibitors of the known blockers of dsRNA-induced apoptosis is used with these cells if dsRNA alone fails to induce apoptosis.

To test for anti-sense thromboxane synthase induction of apoptosis, sense (control) and anti-sense thromboxane synthase are inserted into modified VV vectors and used to infect matched araC-treated glioma cells. Cells are then assayed for induction of apoptosis.

Lung cancer specific mRNAs are identified and anti-sense constructs to those RNAs are used to specifically induce apoptosis in lung cancer cells. Three cell lines, WI-38 cells, A549 cells and A5DC7 cells, are analyzed. WI-38 cells are derived from normal lung tissue, while A549 cells have been derived from a non-small cell carcinoma of the lung. A549 cells are one of a panel of tumor cell lines used by NCI to screen drugs for potential anti-cancer efficacy. A5DC7 cells are a subline of A549 cells, which show an impaired tumor phenotype.

Lung cancer specific RNAs are identified by DD RT-PCR. RNA is isolated from tumor and normal cells according to manufacturer's directions (Gentra, Purescript RNA Isolation Kit, Minneapolis, MN). Briefly, 0.1μg/μl of total RNA is added to four separate reactions, each containing a different oligo-dT primer anchored to the beginning of the poly(A) tail. RT-PCR is used to synthesize random primed segments of cDNA per manufacturers directions (GeneHunter mRNA MAP Kit, Nashville, TN). Briefly, each RT-mix from above is combined with five different AP-primers and tagged with ^{35}S-dATP. A display of the cDNAs is generated in the form of bands on a 6% polyacrylamide/urea gel. Reproducible bands (determined from parallel processing of

two separate mRNA extractions from the two cell populations) that are uniquely expressed in A549 cells but not in WI-38 and A5DC7 cells are excised from the gel. The cDNA is amplified and cloned using a TA Cloning Kit (Invitrogen, San Diego, CA). Differential expression is confirmed by Northern blot analysis or Rnase protection. The nucleotide sequence of the cDNA clone is determined by automated sequencing in the ASU DNA sequencing core facility. Sense and anti-sense cDNA for differentially expressed RNAs are cloned into modified VV, and used to induce apoptosis in araC-treated WI-38, A549 and A5DC7 cells.

CLAIMS

1. A method of inducing apoptosis in a target cell comprising introducing into a target cell an antisense RNA specific for an mRNA in said target cell under conditions whereby said antisense RNA binds to said mRNA to form double-stranded RNA.

2. The method of Claim 1 wherein said target cell is a cancer cell or virus-infected cell.

3. The method of Claim 2 wherein said cancer cell is a lung carcinoma cell, a cervical carcinoma cell, or a rapidly migrating brain cancer cell.

4. The method of Claim 1 wherein said antisense RNA is introduced into said target cell by introduction of a vector comprising a nucleic acid encoding said antisense RNA.

5. The method of Claim 4 wherein said vector is a viral vector.

6. The method of Claim 5 wherein said viral vector is a poxvirus, adenovirus, papilloma virus, parvovirus or vaccinia virus vector.

7. The method of Claim 5 wherein said viral vector is a vaccinia virus vector.

8. The method of Claim 5 wherein said viral vector is a vaccinia virus vector in which the E3L and B13R genes have been detected or inactivated.

9. The method of Claim 5 wherein said viral vector is a vaccinia virus vector in which the E3L, B13R and K3L genes have been deleted or inactivated.

10. The method of Claim 5 wherein said viral vector is a vaccinia virus vector in which the E3L, B13R and G2R genes have been deleted or inactivated.

11. The method of Claim 1 wherein said target cell is a cancer cell and said antisense RNA is specific for multi-drug resistance mRNA.

12. The method of Claim 1 wherein said target cell is a cervical carcinoma cell and said antisense RNA is specific for human papilloma virus E6 or E7 mRNA.

13. The method of Claim 1 wherein said target cell is a rapidly migrating glioma cell and said antisense RNA is specific for thromboxane synthase mRNA.

14. A vaccinia virus vector in which the E3L and B13R genes have been deleted or inactivated, said vector comprising a nucleic acid encoding an antisense RNA that is complementary to an mRNA that is specific to a target cell, wherein said nucleic acid is operably linked to a promoter.

15. The vector of Claim 14 wherein the K3L gene has been deleted or inactivated.

16. The vector of Claim 14 wherein the G2R gene has been deleted or inactivated.

17. The vector of Claim 14 wherein said promoter is a vaccinia virus early promoter.

18. The vector of Claim 14 wherein said promoter is the vaccinia virus E3L promoter.

19. The vector of Claim 14 wherein said antisense RNA is complementary to multi-drug resistance mRNA, human papilloma virus E6 or E7 mRNA, or thromboxane synthese mRNA.

20. A composition comprising the vector of Claim 14 and a carrier.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/03815

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C12N 15/85, 15/86; C07H 21/02, 21/04; A61K 48/00

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 320.1, 325, 375, 455; 536/23.1, 24.31, 24.33, 24.5; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US 5,762,938 A (PAOLETTI et al) 09 June 1998, see entire document.	5-10, 14-18, 20
Y	US 5,639,595 A (MIRABELLI et al) 17 June 1997, col. 4.	11, 19
X	NAORA et al. Antisense sequences of the nbl gene induce apoptosis in the human promyelocytic leukemia cell line HL-60. Leukemia. April 1998, Vol 12, No. 4, pages 532-541, see entire document.	1

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/03815

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SMITH et al. Antisense oligodeoxyribonucleotides targeted to immunoglobulin (IG) sequences in BCL2/IG fusion RNA decrease BCL2 protein expression and induce apoptosis of a t(14;18) positive lymphoma cell line. Blood. December 1995, Vol. 10, Suppl. 1. page 603A.	1
X	MAHDI et al. Combined BCR/ABL abrogation by antisense (AS) oligonucleotides and transfection by the wild type (wt) p53 gene induce apoptosis in K562 cells. Blood. December 1994, Vol. 84, Suppl. 1. page 138A.	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/03815

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 91.1, 320.1, 325, 375, 455; 536/23.1, 24.31, 24.33, 24.5; 514/44